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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of)	
Deborah Kim Glencross)	Group Art Unit: 1651
Application No.: 10/754,711)	Examiner: ALLISON M FORD
Filed: January 12, 2004)	Confirmation No.: 1340
For: CELL ENUMERATION)	

SUBMISSION OF CERTIFIED COPY OF PRIORITY DOCUMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

The benefit of the filing date of the following priority foreign application in the foreign country of South Africa is hereby requested, and the right of priority provided in 35 U.S.C. § 119 is hereby claimed.

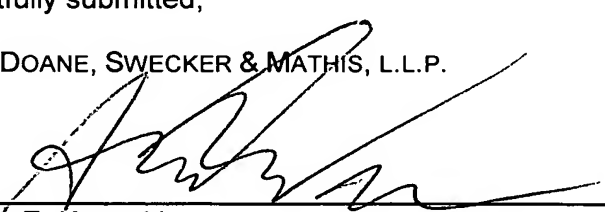
Country: SOUTH AFRICA
Patent Application No.: 2001/5700
Filed: July 11, 2001

In support of this claim, enclosed is a certified copy of said foreign application and which is identified in the original Oath/Declaration Acknowledgement of receipt of this certified document is requested.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

Date: May 16, 2005

By: 
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711

Sertifikaat

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DEPARTEMENT VAN HANDEL
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Certificate

REPUBLIC OF SOUTH AFRICA

PATENT OFFICE
DEPARTMENT OF TRADE AND
INDUSTRY

Hiermee word gesertifiseer dat
This is to certify that

the documents attached hereto are true copies of Forms P2, P6
provisional specification and drawings of South African Patent Application
No. 2001/5700 in the name of National Health Laboratory Service

Filed : 11 July 2001

Entitled : Cell Enumeration

**CERTIFIED COPY OF
PRIORITY DOCUMENT**

Geteken te

PRETORIA

Signed at

in die Republiek van Suid-Afrika, hierdie

in the Republic of South Africa, this

dag van

4th

day of

October 2004

.....
Registrar of Patents

REPUBLIC OF SOUTH AFRICA		REGISTER OF PATENTS		PATENTS ACT, 1978	
OFFICIAL APPLICATION NO.		LODGING DATE: PROVISIONAL		ACCEPTANCE DATE	
22	01	20015700		22	11 JULY 2001
INTERNATIONAL CLASSIFICATION		LODGING DATE: COMPLETE		GRANTED DATE	
FULL NAME(S) OF APPLICANT(S) / PATENTEE(S)					
71	ROUSSOS, Deborah Kim AANSOEKERS VERVANG APPLICANTS SUBSTITUTED				
APPLICANTS SUBSTITUTED:				DATE REGISTERED	
71	NATIONAL HEALTH LABORATORY SERVICE				12/7/2002
UNIVERSITY OF WITWATERSRAND					
ASSIGNEE(S)				DATE REGISTERED	
71					
FULL NAME(S) OF INVENTOR(S)					
72	ROUSSOS, Deborah Kim				
PRIORITY CLAIMED		COUNTRY		NUMBER	
N.B. Use International Abbreviation for country (See Schedule 4)		33	NIL	31	NIL
TITLE OF INVENTION		DATE			
54	"CELL ENUMERATION"				
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PATENT OF ADDITION TO NO.		DATE OF ANY CHANGE			
61					
FRESH APPLICATION BASED ON		DATE OF ANY CHANGE			

REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978
APPLICATION FOR A PATENT AND
ACKNOWLEDGEMENT OF RECEIPT
(Section 30(1) Regulation 22)

IC OF SOUTH AFRICA
FORM P.1
(to be lodged in duplicate)
REVENUE

11.7.01

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THE GRANT OF A PATENT IS HEREBY REQUESTED BY THE UNDERMENTIONED APPLICANT
ON THE BASIS OF THE PRESENT APPLICATION FILED IN DUPLICATE

21 01 PATENT APPLICATION NO 20015700

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54 TITLE OF INVENTION

"CELL ENUMERATION"

Only the items marked with an "X" in the blocks below are applicable.

☐ THE APPLICANT CLAIMS PRIORITY AS SET OUT ON THE ACCOMPANYING FORM P.2. The earliest priority claimed is

Country:

No:

Date:

☐ THE APPLICATION IS FOR A PATENT OF ADDITION TO PATENT APPLICATION NO

21 01

☐ THIS APPLICATION IS A FRESH APPLICATION IN TERMS OF SECTION 37 AND BASED ON

APPLICATION NO

21 01

THIS APPLICATION IS ACCOMPANIED BY:

- ☒ A single copy of a provisional specification of 11 pages
☒ Drawings of 3 sheets
☐ Publication particulars and abstract (Form P.8 in duplicate) (for complete only)
☐ A copy of Figure of the drawings (if any) for the abstract (for complete only)
☐ An assignment of invention
☐ Certified priority document(s). (State quantity)
☐ Translation of the priority document(s)
☐ An assignment of priority rights
☐ A copy of Form P.2 and the specification of RSA Patent Application No
☒ Form P.2 in duplicate
☒ A declaration and power of attorney on Form P.3
☐ Request for ante-dating on Form P.4
☐ Request for classification on Form P.9
☐ Request for delay of acceptance on Form P.4
☐ Extra copy of informal drawings (for complete only)

21 01

74 ADDRESS FOR SERVICE: Adams & Adams, Pretoria

SPOOR & FISHER SANDTON.

Dated this 11 day of July 2001

ADAMS & ADAMS
APPLICANTS PATENT ATTORNEYS

The duplicate will be returned to the applicant's address for service as
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PRETORIA

FORM P6

REPUBLIC OF SOUTH AFRICA
Patents Act, 1978

PROVISIONAL SPECIFICATION

(Section 30 (1) - Regulation 27)

21	01	OFFICIAL APPLICATION NO
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20015700

22	LODGING DATE
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11 July 2001

71	FULL NAME(S) OF APPLICANT(S)
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ROUSSOS, Deborah Kim

72	FULL NAME(S) OF INVENTOR(S)
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ROUSSOS, Deborah Kim

54	TITLE OF INVENTION
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"CELL ENUMERATION"

20015700

THIS INVENTION relates to the enumeration of CD4 + T cells in a cell sample, for example in a bone marrow cell sample, a body fluid sample, a disaggregated tissue sample, a tissue fine needle aspiration sample or, in particular, a blood sample. More particularly, the invention relates to a method suitable for, but not limited to, enumerating the number of CD4 + T cells in a blood sample.

According to the invention, there is provided a method of enumerating the number of CD4 + T cells in a cell sample, the method comprising the steps of:

- counting all the white blood cells in the cell sample to obtain the white blood cell population thereof;

- determining the proportion or percentage of CD4 + T cells in the white blood cell population in the sample; and

- calculating the number of CD4 + T cells in the sample by relating the proportion or percentage of CD4 + T cells to the white blood cell population in the sample, thereby to establish the population of CD4 + T cells in the sample.

Counting the white blood cells (CD45 positive leucocytes) in the sample will naturally involve identifying the white blood cells in the sample, and any suitable known, conventional or established method may be used for such counting (and identification), using any suitable haematology analyser (or flow cytometer). For example, identification of nuclear acid (DNA) staining may be employed as a method of counting the white blood cell population (i.e. the white cell count) in the sample. The white blood cell population in the sample may be defined by flow cytometry, either immunophenotypically as CD45 + cells, or (using a technique substantially similar to DNA staining) as nucleated cells.

Actual calculating of the number of CD4 + T cells in the sample may be performed by multiplying the white blood cell population in the sample by a factor which is the proportion of CD4 + T cells in the white blood cell population when the proportion of CD4 + T cells of the white blood cell population is expressed as a fraction. If the proportion of CD4 + T cells in the white blood cell population is expressed as a percentage, then the white blood cell population will be multiplied by a factor which is a hundredth of the percentage ($\% \times 10^{-2}$).

The invention thus provides a dual-platform method of establishing the number of CD4 + T cells in the sample, using two separate parameters.

Counting the white blood cells in the sample may, as indicated above, be by means of a suitable haematology analyser. Any suitable haematology analyser may be employed, examples being those available under the trade names GenS™, CellDyn™ 4000, and XE2100™, manufactured respectively by Beckman Coulter, Inc. (GenS™), Abbott (CellDyn™ 4000) and Sysmex Corporation (XE2100™). In turn, determining the proportion of CD4 + T cells in the white blood cells in the sample may, as indicated above, be by means of a suitable flow cytometer. Any suitable flow cytometer may be employed, examples being those available under the trade names EPICS™ XL and FACSCalibur™, manufactured respectively by Beckman Coulter, Inc. (EPICS™ XL) and BD Biosciences (FACSCalibur™).

While any suitable technique for counting the white blood cells in the sample may be employed, counting said white blood cells may, for example, be by a size discrimination technique or by an impedance measuring technique.

Similarly, while any suitable technique for determining the proportion of

CD4 + T cells in the white blood cells in a sample may be employed, determining the proportion of CD4 + T cells in the white blood cells in the sample may, for example, be by means of flow-cytometric 90°/side scatter with CD4 expression. While the counting of the white blood cells and the determining of the proportion of CD4 + T cells in the sample may be carried out on separate instruments, namely a haematology analyser and a flow cytometer, it may be possible to combine the functions of such instruments so that both the white blood cell count and the determination of the proportion or percentage of CD4 + T cells of the white blood cells can conveniently be carried out on a single or common instrument.

In a particular embodiment of the invention a dual-platform (i.e. double- or twin-platform) method may be employed, involving two separate instruments to obtain the CD4 + T cell population (i.e. the absolute CD4 + T cell count) in the sample, namely a haematology analyser to obtain the white cell count and a flow cytometer to obtain the fraction or percentage of CD4 + T cells. The white cell count obtained from the haematology analyser is then multiplied by the CD4 + T cell fraction or percentage (as indicated above), measured as a fraction of the population of white blood cells (defined as the CD45 + (positive-expressing) cells) obtained from the flow cytometer. This calculation facilitates the generation of the absolute CD4 + T cell count.

Thus, for example, a Beckman Coulter GenS™ haematology analyser may be used to obtain a white cell count on a blood sample, followed by measurement of the CD4 + T cell fraction or percentage of the white blood cell population (defined by the CD45 + expression thereof) using a Beckman Coulter EPICS™ XL flow cytometer, on the same blood sample.

Instead, a single-platform method may be employed, involving a single

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instrument, the total white cell count and the CD4+ T cell fraction or percentage of the total white cell population being obtained on a single instrument (which may be either a flow cytometer or a haematology analyser). This may be effected by counting the white blood cells directly on a haematology analyser having a facility for fluorescence measurement which can be altered to effect measurement of the fraction or proportion of CD4+ T cells of the total white cell population. Alternatively, the CD4+ T cell fraction or percentage of the total white cell population may be measured directly on a flow cytometer for a blood sample, which flow cytometer has had a suitable (commercially available) bead counting preparation (such as Beckman Coulter Flow-Count™ Fluorospheres) added thereto, the beads being counted simultaneously and being used as a standard from which the absolute number of CD4+ T cells can be calculated.

The invention will now be described, by way of non-limiting illustration, with reference to the following explanatory Examples, with reference to the accompanying drawings, Histograms (I) - (V).

EXAMPLE 1

DUAL-PLATFORM METHOD

A full blood sample was collected from a consenting adult in ethylene diamine tetra-acetic acid (EDTA). This sample was delivered to the laboratory within 6 hours of venesection. Upon receipt in the laboratory the sample was subjected to a white blood cell count performed on a Beckman Coulter GenS™ haematology analyser. A white cell count of $4.80 \times 10^9/\ell$ (liter) was obtained.

Appropriate internal quality control as recommended by the supplier was performed daily on this instrument to ensure that the white cell count was accurate.

After the white blood cell count was obtained, blood taken from the same sample was prepared for flow cytometric analysis. Flow cytometric preparation involved dispensing a well - mixed 100 microlitre (μl) aliquot of whole blood into the bottom of a 12 x 75mm test tube: Anti-CD4 phycoerythrin (PE) monoclonal antibodies and CD45 fluorescein - isothiocyanate (FITC) monoclonal antibodies were added (both obtained from Immunotech, Beckman Coulter, Inc., Miami, Florida) according to the supplier's recommendations and incubated for 10 minutes at room temperature, in the dark.

The tube containing the 100 μl of blood with the added CD45 and CD4 antibodies was then prepared for flow cytometric analysis on a Beckman Coulter Q-Prep / ImmunoPrep™ Reagent System and Workstation, as directed by the supplier. This preparative step included adding a red blood cell lysing agent, a stabiliser and a fixative. The sample was then analysed on a Beckman Coulter EPICS™ XL flow cytometer as set forth hereunder. Appropriate internal quality control including proper alignment and standardisation for light scatter and fluorescence intensity as well as colour compensation as recommended by the supplier was performed daily on this instrument to ensure that the flow cytometry results were accurate.

Thus total leucocytes were first identified on a [CD45 FITC vs. side scatter (SS)] display (Region A in Histogram (I)). All CD45 + gated events in Region A (the total number of leucocytes) were then displayed in Histogram (II), containing only the CD45 + cells, using a [CD4 PE vs. SS] display where CD4 + + low side scatter lymphoid cells, were identified (Region B in Histogram (II)). A CD4+ T cell percentage of 16.5% was noted.

The absolute CD4 + T cell count was obtained by multiplying the white cell count by the CD4 + T cell fraction obtained by flow cytometry i.e. $4.8 \times 10^9/\ell$ multiplied by 16.5% divided by 100. This gave 0.792×10^9 CD4 + T cells/ ℓ (or 792 CD4 + T cells/ $\mu\ell$).

In this method, the total leucocytes served as a common denominator for dual-platform absolute CD4 + T cell counting instead of using the lymphoid population as the common denominator, which, is the state of the art.

The gating strategy employed also made possible the calculation of the CD4 + T cell percentage of total lymphocytes, i.e. the number of events in Gate B (Histogram (II)) divided by the number of events in Gate C. Thus, 752 CD4 + events noted in Gate B were divided by 1416 CD45 + bright events noted in Gate C (Histogram(I)), i.e. $752/1416$ (0.531 or 53.1%). This CD4 cell percentage of total lymphocytes, irrelevant to the present invention, was nevertheless available in case it was clinically relevant, e.g. in paediatric cases.

EXAMPLE 2

SINGLE-PLATFORM METHOD

A full blood sample was collected in EDTA from a consenting adult patient. This sample was delivered to the laboratory within 6 hours of venesection. Upon arrival in the laboratory the sample was prepared for flow cytometric analysis. Flow cytometric preparation involved dispensing (reverse pipetting) a well-mixed 100 microlitre ($\mu\ell$) aliquot of the sample (whole blood) into the bottom of a 12 X 75mm test tube.

Anti-CD4 PE monoclonal antibodies and CD45 FITC monoclonal antibodies were added (both obtained from Immunotech, Beckman Coulter, Inc., Miami, Florida) according to the supplier's recommendations and incubated for 10 minutes at room temperature, in the dark. The tube containing the 100 μ l of blood with the added CD45 and CD4 antibodies was then prepared for flow cytometric analysis using the above mentioned Q-Prep / ImmunoPrep™ Reagent System and Workstation, as directed by the supplier.

After this whole blood preparation step, which included adding a red cell lysing agent, a stabiliser and a fixative, commercially available bead reagents (Flow-Count™ Fluorospheres obtained from Beckman Coulter, Inc., Miami, Florida) were added to the sample as directed by the supplier. This Fluorosphere addition step involved using a very well mixed 100 μ l aliquot of the Flow-Check™ Fluorospheres. The assayed concentration of the Fluorospheres was stated to be 1000/ μ l. The Fluorospheres were added by a reverse pipetting technique with careful attention not to pipette air bubbles. Good reverse pipetting technique was crucial to the accuracy and precision of these test results. The sample was analysed within 2 hours of the addition of the Fluorospheres and was well mixed prior to flow-cytometric analysis.

The sample was then analysed on a Beckman Coulter EPICS™ XL flow cytometer until 1000 Fluorospheres were counted. Appropriate internal quality control including proper alignment and standardisation for light scatter and fluorescence intensity as well as colour compensation, as recommended by the supplier was performed daily on this instrument to ensure that flow cytometry results were accurate. In this analysis total leucocytes were first identified on a [CD45 FITC vs. side scatter (SS)] display (Region A in Histogram (III)). All CD45 gated cells (i.e. gated events) in Region A (the total number of leucocytes) were then displayed in

Histogram (IV), containing only the CD45 + cells, using a [CD4 PE vs. SS] display where CD4 + + , low side scatter lymphoid cells were identified (Region B in Histogram (IV)). The Fluorosphere population was shown in a separate histogram of [Forward Scatter (FS) vs Fluorospheres] the latter of which were detected on log scale of FL4 (fluorescence detector number 4) (Region D, Histogram (V)).

The absolute CD45 + cell count was calculated as directed by the Fluorosphere manufacturer's instructions (Beckman Coulter Flow-Count™), i.e. total number of cells counted divided by the total number of Fluorospheres counted, and then multiplied by the Flow-Count™ Fluorosphere assayed concentration, gave the absolute count/ $\mu\ell$.

The CD45 + leucocyte count was therefore calculated as follows:

The Region A count of 4737 (total CD45 + cells counted) was divided by the Region D count of 1000 (Total Fluorospheres counted), and then multiplied by 1000/ $\mu\ell$ (the Fluorosphere assayed concentration), which gave 4737/ $\mu\ell$ or $4.737 \times 10^9/\ell$ (the absolute CD45 + leucocyte or white cell count). The absolute CD4 + T cell count was obtained by multiplying this calculated CD45 + white cell count by the percentage of CD4 + + , low side-scatter T cells counted within the CD45 + leucocyte population (Region B, Histogram (IV)) i.e 4737/ $\mu\ell$ multiplied by 16.2% (i.e. by 0.162). This gave 766 CD4 T cells/ $\mu\ell$ (or 0.766×10^9 CD4 + T cells/ ℓ).

In this method, the total CD45 + leucocyte count served as the base line for the absolute CD4 + T cell counting instead of using the lymphoid population or CD3 positive lymphocyte count as the base line, which is the state of the art.

The gating strategy employed also made possible the calculation of the CD4 + T cell percentage of total lymphocytes i.e. the number of cells in Gate B (Histogram

(IV)) divided by number of cells in Gate C (Histogram III)). Thus, 766 events noted in Gate B were divided by 1421 CD45+ bright events noted in Gate C, i.e. 766 divided by 1421 (0.539 or 53.9%). This value was not however used in the generation of the CD4+ count, and was merely extra information available in case the CD4 percentage of lymphocytes was clinically relevant, e.g. in paediatric cases.

Regarding Histograms (I) - (IV), it is to be noted that Histogram (I) is identical to Histogram (III)), and Histograms (II) and (IV) are identical to each other, with the exception of the presence of beads in Histogram (III).

Advantages of the invention, particularly as described with reference to the above Example(s), are that the use of a count of the white blood cell population incorporating the use of CD45 expression as the basis for calculating the number of CD4+ T cells in cell samples facilitates accurate determinations after delays of up to several days after sample collection. Both CD45 and CD4+ T cell expressions are preserved with only minor losses of fluorescence intensity for up to 48 - 60 hours, even after loss of forward scattering properties. Indeed both the CD45+ leucocyte- and the side-scattering features of white blood cells promise to be retained for up to 5 days after sample collection. By virtue of including both the CD45+ leucocyte- and side- scattering parameters, technical errors can be avoided and irrelevant cellular events arising from, for example, monocytes or red blood cells, can be excluded while relevant lymphoid cells e.g. those with apoptotic scatter features, can be included. The approach of the present invention is particularly amenable to paediatric samples where small quantities of blood are available. In the absence of CD45+ leucocyte staining, unlysed and nucleated red blood cells may drastically interfere with the definition of both CD4+ T lymphocyte absolute counts and CD4+ T lymphocyte fraction- or percentage values. In infants and children the CD4+ T lymphocyte percentage counts are used as the clinically

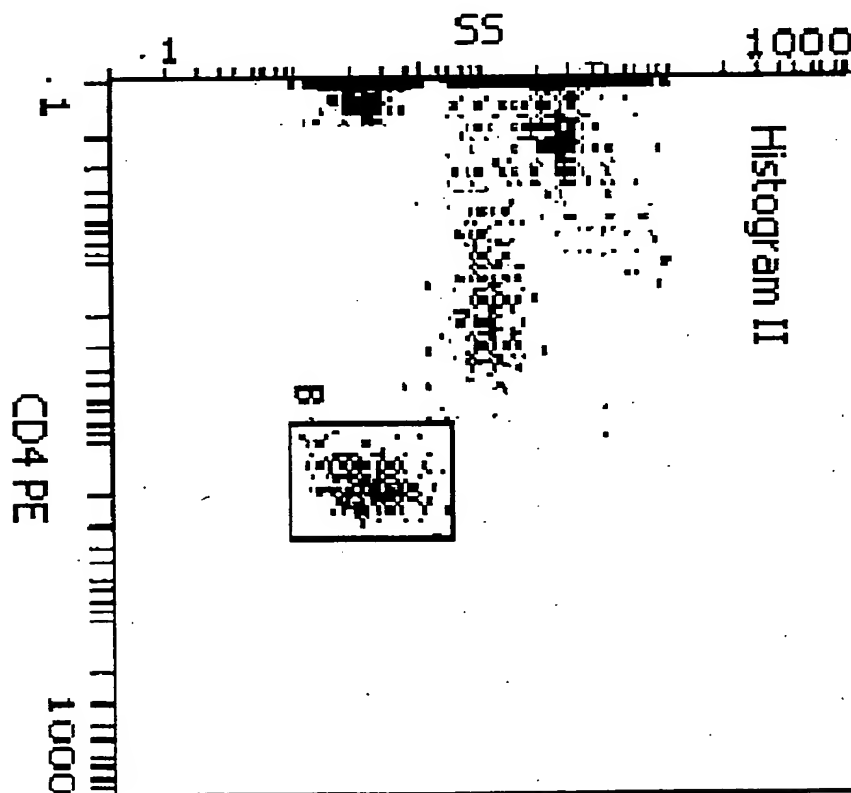
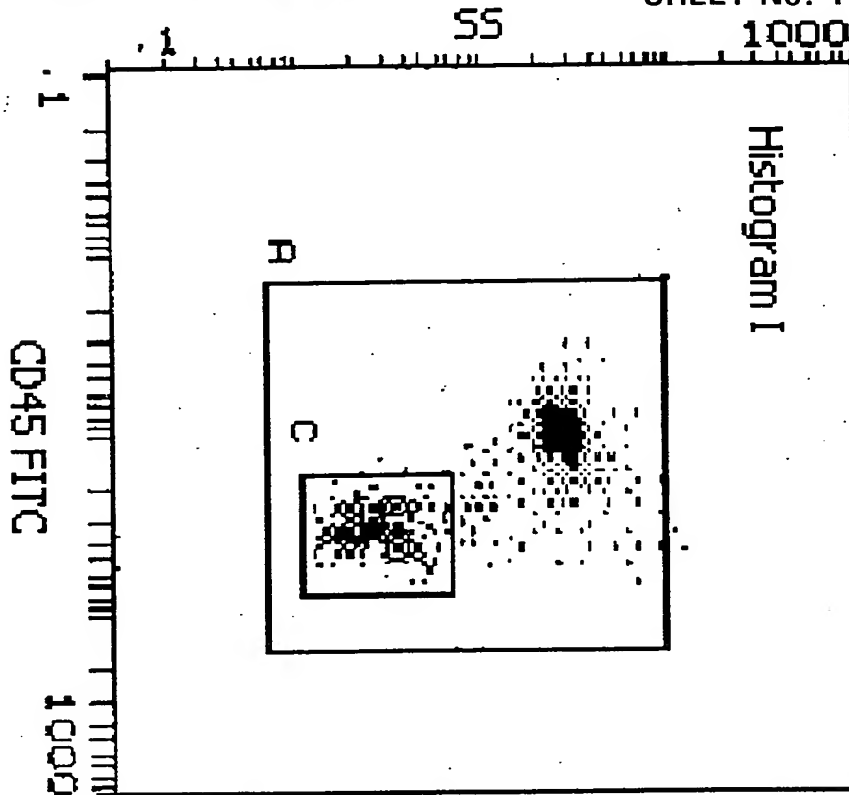
relevant parameter because of the age-dependent variability of the CD4+ T absolute counts. Use of CD45+ leucocyte counts as a basis facilitates use of precise CD4+ T lymphocyte fraction- or percentage values by identifying the CD4+ + T cells as a function the bright CD45+ + leucocyte cells.

The method of the present invention is relative robust, reproducible and accurate while being easy to use and comparatively inexpensive and easily implemented.

DATED THIS 11th DAY OF JULY 2001

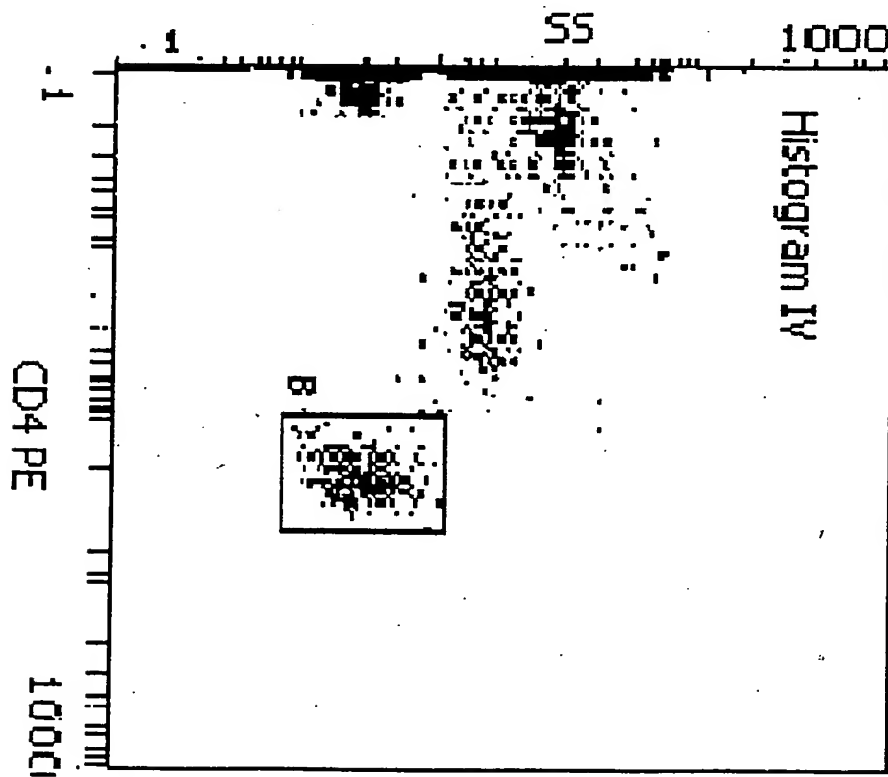
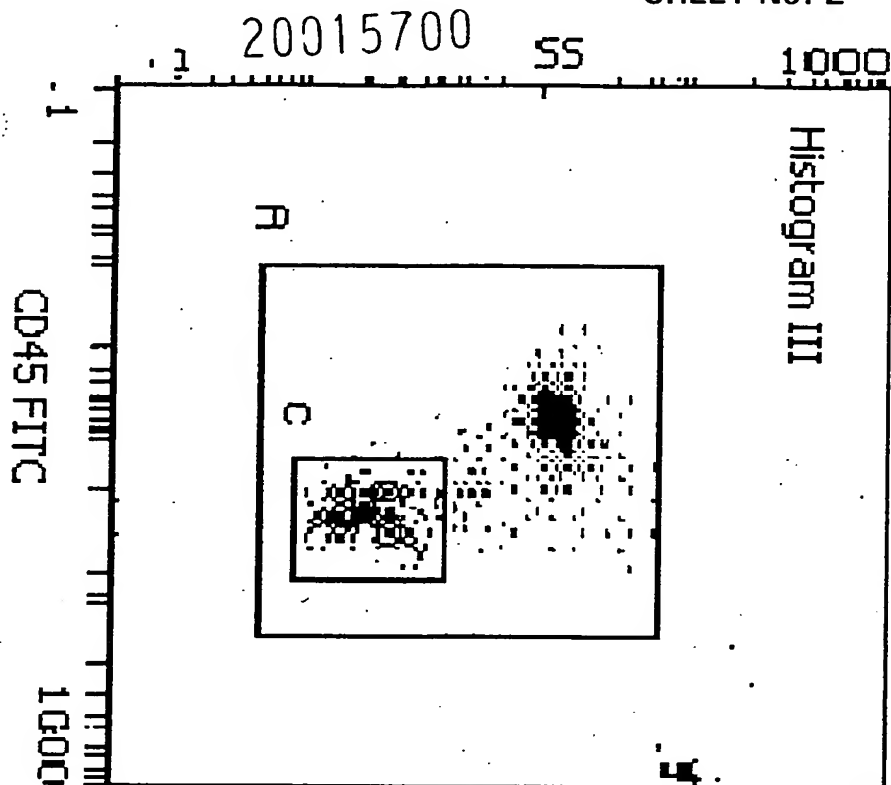


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APPLICANT'S PATENT ATTORNEYS



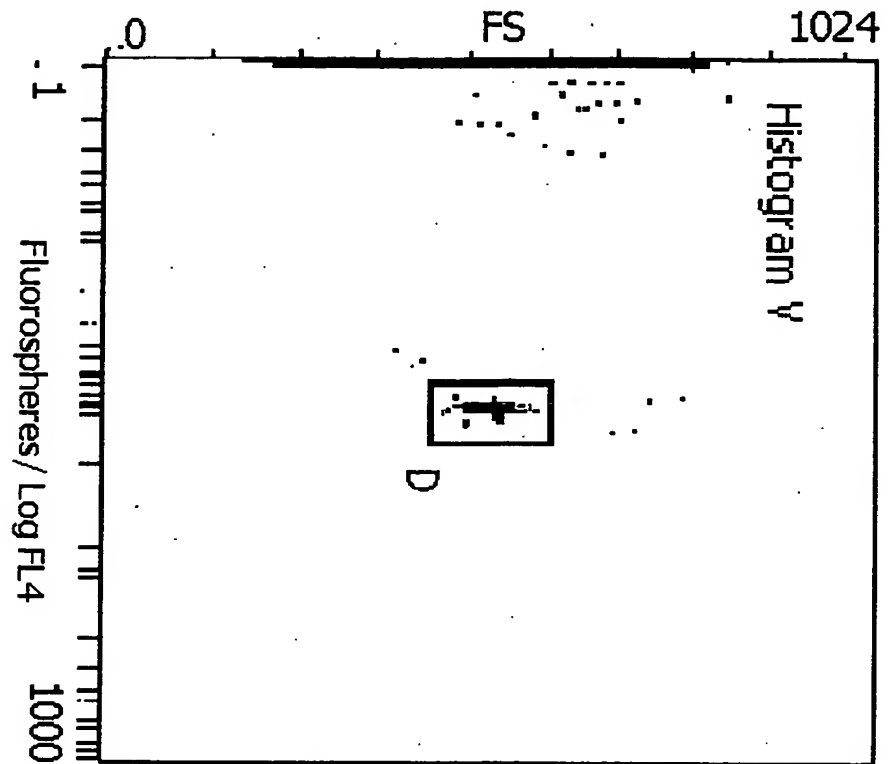
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